



Arbuscular mycorrhizal fungus *Rhizophagus irregularis* enhances antioxidant system mechanisms in chickpea plants under saline conditions

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Abstract

Under soil saline conditions, biochemical alterations occur during plant-arbuscular mycorrhizal fungi (AMF) interactions, depending on the fungal strain involved and the intensity of the stress. This study aimed to evaluate the infectivity and efficacy of *Rhizophagus irregularis* by analyzing the biochemical responses it induces in chickpea (*Cicer arietinum* L.) cv. ‘Nacional-29’ under 50 mM NaCl stress. Nacional-29 is a chickpea variety developed at the Institute of Fundamental Research in Tropical Agriculture (INIFAT) “Alejandro de Humboldt” and is classified as susceptible to salinity when the soil saturation extract exceeds 30 mM. The effect of an AMF strain (INCA 11 of *Rhizophagus irregularis*) from the INCA collection was assessed in chickpea plants regarding root colonization, accumulation of cytocompatible osmolytes, and the activity of various antioxidant enzymes. The analysis demonstrated enhanced antioxidant defense mechanisms, a significant increase in proline accumulation in the leaves of stressed mycorrhizal plants, and a substantial reduction in lipid peroxidation, with levels even lower than those in the inoculated control plants. The applied salt stress caused a redox imbalance, leading to a reduction of POX, SOD, and APX by 35%, 20%, and 25%, respectively, while increasing free radical levels. However, the inoculated stressed plants exhibited higher levels of total soluble carbohydrates and increased catalase and glutathione reductase activity, indicating that redox homeostasis was crucial for salinity tolerance. These findings indicate that *R. irregularis* enhances antioxidant defense mechanisms in chickpea plants, thereby improving their salinity tolerance. This could have significant implications for agricultural practices, particularly in developing strategies to enhance crop resilience in saline soils. Arbuscular mycorrhizal fungi, such as *R. irregularis*, provide a sustainable solution for enhancing crop productivity in saline-affected areas, thereby contributing to food security in regions facing soil salinity challenges.

Keywords Salinity · Antioxidant enzymes · Osmolytes · Mycorrhizae

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Introduction

Soil salinity is one of the most critical edaphic factors limiting crop growth and productivity. Over 20% of global agricultural soils are affected by salinity (Liu et al. 2022). Soil salinization poses a major threat to land productivity, impacting approximately 1 billion hectares across more than 100 countries (Khasanov et al. 2023). Soil salinity adversely affects several crops, including cotton, rice, and wheat, leading to reduced plant growth and yields (Chaudhary et al. 2024). Under saline conditions, plants activate various biochemical and physiological mechanisms to adapt and survive. These include changes in morphology, anatomy, water relations, photosynthesis, hormone profiles, ion distribution, and antioxidant system responses (Acosta et al. 2017). Salt stress affects numerous economically and nutritionally important crops, such as chickpea (*Cicer arietinum* L.), which is the second most important legume worldwide after beans (*Phaseolus* spp.). However, this crop is highly sensitive to salinity, which negatively affects germination (Khalid et al. 2001), vegetative growth (Khan et al. 2015), and reproductive processes (Pushpavalli et al. 2015; Khan et al. 2017).

The association of plants with beneficial soil microorganisms, such as vesicular-arbuscular mycorrhizal (VAM) fungi, has been studied since the 1960s for its role in enhancing plant salt tolerance.

Arbuscular mycorrhizal fungi (AMF) can be an alternative for mitigating the adverse effects of salinity and decreasing crop productivity under these conditions (Pozo et al. 2015; Rivero et al. 2018). AMF contribute to improve plant salt tolerance through several ways, for example, enhanced antioxidant enzyme activity, reduced reactive oxygen species levels, and the maintenance of water regime. AMF can also induce molecular mechanisms that enhance the host's salt tolerance through gene expression changes (Han et al. 2024).

AMF naturally occur in saline soils and contribute to soil stability and plant adaptation in stressful environments. They enhance plant growth through improved nutrient uptake (Evelin et al. 2019) and help maintain enzymatic processes and protein synthesis (Parvin et al. 2020). AMF under salt stress conditions can maintain cellular homeostasis, enhance water uptake and contribute to osmotic balance in plants, activate antioxidant systems to prevent damage caused by reactive oxygen species (ROS) formation, protect the photosynthetic apparatus (Elhindi et al. 2017;

Ait-El- Mokhtar et al. 2019) and regulate hormone profiles to minimize the effects of salt on plant growth and development (Begum et al. 2019).

The application of AMF in saline soils presents a promising strategy for increasing the production of grains such as chickpeas, thereby enhancing the profitability of saline-affected soils and contributing to food security in developing countries facing salinization problems (Tavares et al. 2024). In this context, a study was conducted to evaluate the effect of mycorrhizal inoculation on the biochemical and physiological indicators of the chickpea (cultivar Nacional-29) under saline conditions. Nacional-29 is a chickpea variety developed at the Institute of Fundamental Research in Tropical Agriculture (INIFAT) 'Alejandro de Humboldt' and is classified as susceptible to salinity when the electric conductivity of the soil saturation extract exceeds 30 mM. Its average yield under non-saline soil conditions is 2.3 t ha⁻¹, with a biological cycle of 125 days.

This study is the first to demonstrate that *Rhizophagus irregularis* enhances redox homeostasis in salt-sensitive chickpea cultivar (*Cicer arietinum* cv. 'Nacional-29'), providing new insights into its biochemical role beyond nutrient uptake.

Materials and methods

The experiment was conducted under controlled conditions at the Laboratory of Plant Physiology, Technological Institute of Sonora, Mexico. The seeds were pre-germinated in distilled water (electrical conductivity: 0.14 dS m⁻¹) for 24 h. After treatment, the seeds were sown (one per pot) in 5 L polypropylene pots filled with compacted, non-sterile Fluvisol soil, which had an electrical conductivity of 0.75 dS m⁻¹ at saturation (Table 1). According to some experiences, the use of this type and volume of pots supports the development of chickpea plants (Mitache et al. 2024). The pots were pierced with 5 holes, each with a diameter of 3 mm, to prevent waterlogging.

The soil used to fill the pots was taken from land that had been cultivated with wheat for over 30 years. The area where the soil was taken was chosen because there are no records of chickpea or other legume cultivation in the last 30 years. This soil is located near the lower canal of the Yaqui Valley irrigation district in Ciudad Obregón, Sonora, Mexico. This soil classification correlates with the Fluvisol group according to the WRB (2015). This type of soil is

Table 1 Chemical properties of the soil used in the experiment. [The values are the average of nine samples well randomized taken in the experimental area. E.C: electric conductivity; TSS, total soluble salts; OM, organic matter. pH: Hydrogen potential in water]

Exchangeable cations (cmol _c kg ⁻¹)				P ₂ O ₅ (mg kg ⁻¹)	EC (dS m ⁻¹)	TSS (mg kg ⁻¹)	OM (%)	pH (H ₂ O)
Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺					
1.4	1.13	6.9	4.17	238.75	0.75	537	3.23	7.4

suitable for chickpea cultivation (Dutta et al. 2024) mainly due to its good internal and surface drainage, which guarantees good crop development without the appearance of diseases associated with over-humidity.

The treatments were distributed in a completely randomized design, for a total of four treatments. Each treatment had 40 plants, one plant per pot for a total of 160 plants in the experiment.

The treatments were as follows:

1. Control (0 mM NaCl).
2. Salinized (50 mM NaCl).
3. *R. irregularis*.
4. *R. irregularis* + 50 mM NaCl.

Plants were cultivated under controlled conditions of 26/20 °C (day/night), a 12-hour light/dark photoperiod, and 80% relative humidity. At 15 days after sowing the seeds, salt stress was applied twice a week for 35 days, after which 50 mL of saline solution was added according to the salinity treatments, and 50 mL of water was added to the control treatments. The selection of 50 mM NaCl was based on the fact that in regions where chickpea cultivation is common, the maximum electrical conductivity of the soil saturation extract is 50 mM. This value most affects the physiological and agronomic performance of the crop. The soil was maintained at a humidity percentage of 85% throughout the cultivation to avoid the combination of the water stress factor with the induced salinity and also to maintain a constant electrical conductivity of 50 mM in each pot. The inoculation of *R. irregularis* in the pots was carried out at the time of sowing by depositing a mass of 2 g per pot (45–50 spores g⁻¹) (Meriño-Hernández et al. 2022).

Mycorrhizal variables

At the end of the experiment (35 days after sowing), mycorrhizal variables were evaluated. Root colonization and visual density: For both variables, samples of the finest roots were collected from 10 plants per treatment. Approximately 200 mg of secondary or tertiary roots were selected for staining according to the methodology described by Rodríguez and Sanders (2015). The colonization percentage was quantified by examining 100 visual fields under a stereomicroscope (NOVEL N-180 M, Nanjing Jiangnan Novel Optics Co., Ltd.; China) using the intercept method developed by Giovanetti and Mosse (1980). The results were transformed using the arcsen $\sqrt{x}$ function, as described by Lerch (1977). The mathematical calculation of visual density was performed using the methodology described by Trouvelot et al. (1986).

Biochemical variables

The plants were evaluated before the end of the experiment to determine the mechanisms underlying the effects of NaCl stress on mycorrhizal chickpea plants. These mechanisms include the contents of osmotically active organic solutes, certain enzymatic components of oxidative metabolism, and the levels of hormones such as abscisic acid and gibberellic acid.

Osmotically active organic solutes

The contents of proline and total soluble carbohydrates in leaves and roots, as well as glycine betaine and glutamate in leaves, were determined. For this, three samples were taken from a mixture of the third, fourth, and fifth pairs of leaves from five plants per treatment (Yoshida 1981). For root determination, three samples were taken from a mixture of the whole roots of the same five plants. Each sample had three laboratory replicates ($n=9$). Extracts were obtained using a refrigerated centrifuge (Eppendorf 5804 R), and a UV/visible spectrophotometer (Genesys 6, Thermo Electron Corporation) was used for colorimetric determination.

Proline content was determined by reaction with ninhydrin (Bates et al. 1973). For this, three 0.25 g samples of plant tissue (leaf and root) were taken, frozen in liquid nitrogen, and homogenized with 10 mL of distilled water at 100 °C. The samples were then filtered, and 2 mL of the extract was reacted with 2 mL of glacial acetic acid and 2 mL of ninhydrin at 100 °C for one hour. The reaction was stopped in an ice bath, and the proline chromophore was extracted with 4 mL of toluene. Proline was quantified at 520 nm using L-proline (SIGMA) for the calibration curve.

Glycine betaine was synthesized following the method proposed by Grieve and Grattan (1983), based on the reaction of glycine betaine with KI-I₂. For this, 0.25 g of leaves, previously dried for 72 h, were removed, extracted in 10 mL of deionized water, shaken for 48 h, and filtered. A 0.5 mL aliquot was taken, diluted 1:1 with NH₂SO₄, vortexed gently, and allowed to stand on ice for one hour. Then, 0.1 mL of cold KI-I₂ was added, and the samples were capped and refrigerated at 0–4 °C for 16 h. The samples were centrifuged at 16,000 rpm for 15 min at 0 °C, and the supernatant was collected. Subsequently, 4.5 mL of 1,2-dichloroethane was added, and the samples were incubated at room temperature for 2.5 h. Finally, 1 mL of the supernatant was removed, and absorbance was measured at 365 nm. The values obtained were interpolated using the calibration curve made with a betaine standard (SIGMA).

Glutamate (GLU) activity was determined by measuring the activity of the enzyme GS, following the method of O'Neal and Joy (1973). A 0.5 g sample of fresh leaves was

homogenized with 5 mL of 0.2 M HEPES buffer (pH 7.9). The homogenate was centrifuged at 16,000 rpm for 20 min, and the supernatant was used to measure GS activity. Enzyme activity was determined by the method described by Slawky and Rodier (1988). To determine GLU concentrations, an enzyme omission blank was used, in which the corresponding volumes were replaced with HEPES buffer. The values were expressed as mg g^{-1} FW.

Total soluble carbohydrates were determined using the method proposed by Yemm and Willis (1954). For the preparation of the extracts, three 0.25 g samples of leaves and roots were taken from each treatment and macerated with liquid N₂. Three milliliters of 80% (v/v) ethanol were added, and the mixture was heated in a water bath at 85 °C for 30 min. The samples were then centrifuged at 16,000 rpm for 10 min, and the supernatant was collected. This procedure was repeated three times. The solvent from the supernatants was evaporated in a sand bath to a final volume of 3 mL, and the volume was brought to 10 mL with distilled water. For determination, 0.5 mL of the diluted sample (1:50) was placed on ice. Anthrone (2%) diluted in concentrated H₂SO₄ was slowly added, and the mixture was heated in a water bath at 100 °C for 7.5 min. After cooling on ice, the mixture was incubated at room temperature for 5 min. Absorbance was measured at 630 nm. The values were interpolated using the calibration curve made with different concentrations of glucose (SIGMA) and are expressed in $\mu\text{g glucose g}^{-1}$ FW.

Oxidative metabolism

In this case, lipid peroxidation and the enzymatic activity of several antioxidant enzymes in leaves were evaluated. For this purpose, plant extracts were first obtained from three 0.25 g samples of leaf tissue from each treatment and extracted with 2.5 mL of a solution of potassium phosphate buffer (0.1 mol L⁻¹, pH 7.8), 0.1 mmol L⁻¹ EDTA, 0.1% (v/v) Triton X-100, 1.5% (w/v) polyvinylpyrrolidone (PVPP) and 2.5 $\mu\text{g L}^{-1}$ of the protease inhibitors aprotinin and leupeptin. The homogenate was centrifuged at 16,000 rpm for 20 min at 4 °C, and the supernatant was collected for further analysis.

Lipid peroxidation

This variable was estimated by determining the malondialdehyde (MDA) content (Hodges et al. 1999). For the analysis, 500 μL of the supernatant was mixed with the same volume of 0.5% (w/v) thiobarbituric acid (TBA) dissolved in 20% (w/v) trichloroacetic acid (TCA). The mixtures were heated at 95 °C for 25 min in 1.5 mL Eppendorf tubes with holes in the lid to allow the vapors to escape. Subsequently, they

were cooled on ice for 15 min and centrifuged at 16,000 rpm for 10 min at 4 °C. The absorbance was measured at 532 and 600 nm. The absorbance at 600 nm was subtracted from the reading at 532 nm to discount the nonspecific absorbance. The amount of MDA was determined using the extinction coefficient of 155 $\text{mM}^{-1} \text{cm}^{-1}$, expressed as the amount of thiobarbituric acid reactive substances (TBARS) per gram of fresh tissue mass.

Antioxidant enzyme activity

Peroxidase (POX, EC 1.11.1.7)

For the determination of enzyme activity, 50 μL of the collected supernatant was mixed with 0.1 mol/L phosphate buffer (pH 7), 1% guaiacol (v/v) and 0.25% hydrogen peroxide (v/v) in a total volume of 1 mL. The concentrations of guaiacol and H₂O₂ were standardized prior to the assays. The formation of oxidized guaiacol was measured by monitoring the increase in the absorbance of tetraguaiacol at 470 nm every 5 s over a 30 s interval (Chance and Maehly 1955). The specific POX activity was calculated using the extinction coefficient of guaiacol (26.6 $\text{mM}^{-1} \text{cm}^{-1}$) and is expressed as $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{mg}^{-1}$ protein.

Catalase (CAT, EC 1.11.1.6)

The enzyme activity was determined using 50 μL of the supernatant in a reaction mixture containing 50 mmol L⁻¹ phosphate buffer (pH 7.0) and 10 mmol L⁻¹ hydrogen peroxide in a volume of 1 mL. The decomposition of H₂O₂ was evaluated every 10 s for 1 min, and the amount of H₂O₂ was estimated by the absorbance at 240 nm. CAT activity was calculated using the extinction coefficient of H₂O₂ (0.0436 $\text{mM}^{-1} \text{cm}^{-1}$) and expressed as $\mu\text{mol of H}_2\text{O}_2 \text{ min}^{-1} \text{mg}^{-1}$ protein (Aebi 1984).

Superoxide dismutase (SOD, EC 1.15.1.1)

The enzyme activity was assayed by nitrotetrazolium blue (NBT) reduction (Beyer and Fridovich 1987). In the assay, 50 μL of the supernatant was added to a reaction mixture containing 50 mmol L⁻¹ bicarbonate buffer (pH 10.2), 13 mmol L⁻¹ methionine, 0.1 mmol L⁻¹ EDTA and 63 $\mu\text{mol L}^{-1}$ NBT. The reaction was carried out in glass tubes in a final volume of 4 mL, and the reaction was started with 1.3 $\mu\text{mol L}^{-1}$ riboflavin and exposed to white light (15 watts, fluorescent lamp) for 2 min. The reaction was stopped in the dark. Unilluminated blanks were used for each sample. The amount of NBT was estimated by the absorbance at 560 nm. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the photoreduction of

NBT. The specific SOD activity was expressed as SOD AUE mg^{-1} prot.

Ascorbate peroxidase (APX, EC 1.11.1.11)

Fifty microliters of the supernatant was added to a reaction mixture containing 50 mmol L^{-1} phosphate buffer (pH 7.0), 50 mmol L^{-1} ascorbic acid, 0.1 mmol L^{-1} EDTA and 0.1 mmol L^{-1} hydrogen peroxide in a final volume of 1 mL (Nakano and Asada 1981). Monitoring of H_2O_2 dependent ascorbate oxidation was assessed 10 times at 100-second intervals by the decrease in absorbance at 290 nm. The specific APX activity was calculated using the extinction coefficient of ascorbate ($2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as $\mu\text{mol Asc min}^{-1} \text{ mg}^{-1}$ protein.

Glutathione reductase (GR, EC 1.6.4.2)

This enzyme catalyzes the formation of reduced glutathione from oxidized glutathione. The reaction mixture contained, in a total volume of 1 mL, Tris-HCl buffer (50 mmol L^{-1} , pH 7.5), MgCl_2 (5 mmol L^{-1}), NADPH (0.2 mmol L^{-1}), oxidized glutathione (GSSG) (0.5 mmol L^{-1}) and 50 μL of the supernatant, which initiated the reaction. The absorbance was measured every 20 s for 3 min at 340 nm (Carlberg and Mannervik 1985). GR activity was calculated using the extinction coefficient of NADPH ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ of protein.

Absciscic acid (ABA) and gibberellic acid (GA_3) contents

For leaf extraction, 3 g of the sample was taken, and 40 mL of 80% (v/v) methanol containing 4 mg of 2,6-di-tert-butyl-4-methylphenol was used. The extracts were then centrifuged at $10,000 \times g$ for 20 min at 4 °C, and the supernatants were evaporated at 40 °C on a rotary evaporator. The residues were solubilized by adding 10 mL of petroleum ether followed by 10 mL of 0.02 M phosphate buffer solution (pH 8.0). Then, 0.2 g of insoluble (crosslinked) PVPP was added

to the solutions and mixed at 0 °C for 15 min. The PVPP was then removed by vacuum filtration. Ethyl acetate (pH 3.0) was added to the solutions, and the upper layers were removed and evaporated to dryness at 40 °C. Each residue was dissolved in 1 mL of 50% (v/v) methanol for HPLC analysis. Aliquots of each sample (2 μL) were separated by HPLC (1200 Series; Agilent Technologies, USA) on a $4.8 \times 150 \text{ mm}$ C18 column (Agilent Technologies) at a flow rate of 0.8 mL min^{-1} . The solvents used were 0.8% (v/v) glacial acetic acid (solvent A) and 100% methanol (solvent B). The hormones were eluted from the column using a varying gradient of solvent B. The retention times of the hormones were determined with two commercial standards, ($\pm$)-abscisic acid (A1049, Sigma, St Louis, MO, USA) and gibberellic acid (gibberellin A_3 , Sigma), at a wavelength of 260 nm. The results are expressed as $\mu\text{g g}^{-1}$ FW.

Statistical analysis

After confirming that the obtained data met the theoretical assumptions of normality and homogeneity of variance, as assessed by the Kolmogorov-Smirnov, Cochran, Hartley, and Bartlett tests, a one-way ANOVA was performed. When significant differences were detected between treatments, Duncan's multiple range test, also known as the Cochran and Cox test, was applied with a significance level of $p \leq 0.05$ using IBM SPSS Statistics version 22.

Results and discussion

Mycorrhizal variables

Mycorrhizal colonization and visual density data are presented in Fig. 1. In both parameters, higher values were observed in the inoculated treatments compared to the non-inoculated ones. These results indicate that the applied stress did not negatively affect mycorrhizal colonization or visual density in inoculated plants. Although there were no

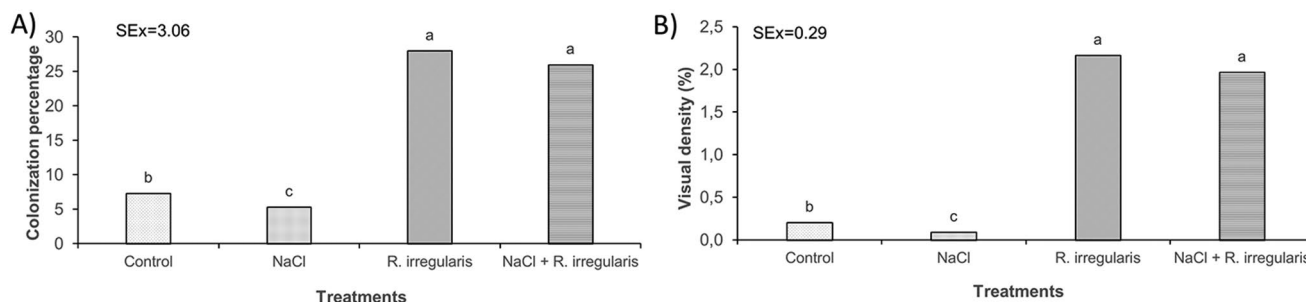


Fig. 1 **A)** Mycorrhizal colonization and **B)** visual density in roots of chickpea cv. 'N-29' plants inoculated with *R. irregularis* and exposed to 50 mM NaCl, 35 days after sowing. Means with the same letters do

not differ significantly from each other according to Duncan's multiple range test ($p \leq 0.05$) ($n=3$). SE: Standard error of the mean

significant differences between the *R. irregularis* and 50 mM NaCl+*R. irregularis* treatments, a slight decrease in colonization percentage was observed. This result is consistent with Xu et al. (2024), who observed a reduction in colonization percentage with increasing salinity in alfalfa (*Medicago sativa* L.) under saline conditions.

The lowest values were observed in the 50 mM NaCl treatment. The low mycorrhizal colonization and visual density observed in the roots of non-inoculated plants were due to the presence of AMF in the soil, which remained below 5% in all cases.

In a study, Garg and Bhandari (2016a) detected an increase in mycorrhizal colonization by *F. mosseae* on chickpea plants exposed to 60, 80, and 100 mM NaCl in both sensitive and tolerant genotypes. In contrast, Garg and Bharti (2018) reported a gradual decline in root colonization of chickpea inoculated with *R. intraradices* as NaCl concentrations increased, with different values observed between the susceptible and tolerant genotypes. Other research conducted under salt stress conditions has shown a decrease in mycorrhizal colonization in the roots of wheat (*T. aestivum*) (Zhu et al. 2016) and acacia (*Acacia arabica*) (Datta and Kulkarni 2014). The results of this study suggest that mycorrhizal colonization may exhibit varying responses to NaCl stress, depending on factors such as genotype, AMF strain, severity of the applied stress, and the substrate used.

Biochemical variables

Osmotically active organic solutes

Table 2 shows the proline content in leaves and roots and glycine betaine and glutamate in chickpea leaves exposed to salinity and inoculated with *R. irregularis*. Salt stress led to an increase in proline concentration in chickpea leaves under both salinity treatments (50 mM NaCl and 50 mM NaCl+*R. irregularis*), with no significant differences between them.

Table 2 Effects of mycorrhizal inoculation with *R. irregularis* on leaf and root proline (PRO), glycine betaine (GB) and glutamate (GLU) contents in the leaves of Chickpea Cv. 'N-29' plants exposed to 50 mM NaCl 35 days after sowing

Treatments	PRO (Leaves) ($\mu\text{mol g}^{-1}$ MF)	PRO (roots) ($\mu\text{mol g}^{-1}$ MF)	GB ($\mu\text{g g}^{-1}$ MF)	GLU (mg g^{-1} MF)
Control	17.61 b	2.96 b	19.20 c	7.64 c
50 mM NaCl	23.32 a	5.07 a	24.50 a	14.17 a
<i>R. irregularis</i>	14.53 c	2.95 b	17.60 d	10.23 b
<i>R. irregularis</i> + 50 mM NaCl	24.00 a	3.27 b	22.37 b	11.46 b
SE _x	$\pm 1.23^{***}$	$\pm 0.29^{***}$	$\pm 0.82^{***}$	$\pm 0.70^{***}$

Means with equal letters in each column do not differ significantly from each other according to Duncan's multiple range test ($p \leq 0.05$) ($n=9$). SE_x: Standard error of the mean

In contrast, in roots, mycorrhizal inoculation decreased the concentration of this osmolyte compared to that in non-inoculated stressed plants. On the other hand, glycine betaine accumulation had a similar trend to that of proline.

The increase in proline content in leaves under saline conditions is closely linked to higher Na accumulation in this organ (Ubaidillah et al. 2024). Consequently, salt stress resulted in increased proline levels in both inoculated and non-inoculated stressed plants. However, the increase in proline concentration of mycorrhizal plants was nearly double that of nonmycorrhizal plants, with increases of 65 and 32%, respectively, indicating the greater potential of plants inoculated with *R. irregularis* to perform osmotic adjustment, as well as the effectiveness of this strain (INCAM 11) in the salinity tolerance response of chickpea cv. 'N-29'.

Salt stress tolerance mechanisms often involve proline accumulation. This osmolyte functions as a compatible organic solute and osmoprotectant (Hameed et al. 2014), playing a crucial role in stabilizing cellular proteins and membranes under high salt conditions.

Several authors have reported that the concentration of this osmolyte is greater in mycorrhizal plants than in nonmycorrhizal plants at different salinity levels (Garg and Pandey 2016; Froisi et al. 2018; Hashem et al. 2018). Similar proline concentration values to those in this study were found in chickpea cultivated with AMF under saline conditions (Arefian et al. 2014). On the other hand, glycine betaine had a similar trend to that of proline (Table 2). The concentrations of this osmolyte found in chickpea leaves under both salt stress conditions increased, and although mycorrhizal plants had a lower value, the increase was similar (approximately 27%) in both mycorrhizal and non-mycorrhizal plants.

Fariduddin et al. (2013) have described that in plants subjected to salt stress, glycine betaine contributes to the preservation of osmotic balance through its role as a cyto-compatible solute, stabilizes the structure of proteins such as Rubisco and Rubisco activate, protects the photosynthetic apparatus, especially the reaction centers, and can act as a free radical scavenger.

The foliar concentration of glutamate, another amino acid, increased under saline conditions (Table 2); however, a slight increase was detected in mycorrhizal plants compared with non-mycorrhizal plants. A greater percentage of non-inoculated stressed plants (50 mM NaCl) showed an increase of 85%; in contrast, inoculated stressed plants (50 mM NaCl+*R. irregularis*) exhibited lower percentages with an increase of 12%. On the other hand, the control plants had lower concentrations of this amino acid.

Figure 2 shows the total soluble carbohydrate content in the leaves and roots of chickpea plants subjected to NaCl stress. Salt stress increased total soluble carbohydrate

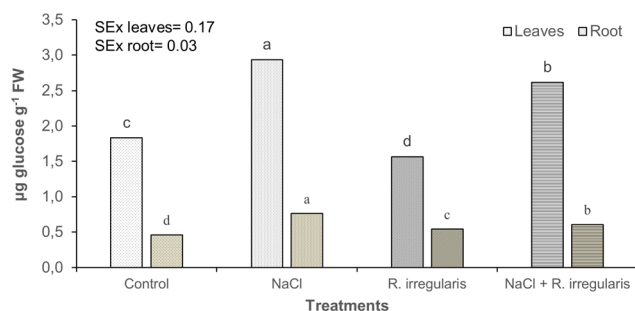


Fig. 2 Effects of mycorrhizal inoculation on the total soluble carbohydrate content in the leaves and roots of chickpea cv. 'N-29' plants exposed to 50 mM NaCl 35 days after sowing. Means with the same letters do not differ significantly from each other according to Duncan's multiple range test ($p \leq 0.05$) ($n=9$)

concentration in both organs, with higher levels in non-inoculated plants.

Similar results were reported by Frosi et al. (2018) for *Cenostigma midale* plants inoculated with *Acaulospora longula* and *C. etunicatum*. In contrast to the results of this study, Evelin et al. (2013) reported higher levels of glycine betaine and total soluble carbohydrates and lower levels of proline in mycorrhizal fenugreek (*T. foenum-graecum*) plants exposed to NaCl stress.

The results of the variables related to osmotically active solutes showed an increase in the leaves of chickpea plants exposed to NaCl stress in most cases (glycine betaine, glutamate and total soluble carbohydrates) in non-inoculated plants, which is indicative of the greater degree of stress in these plants. In the case of proline, the greatest increase was found in the plants with mycorrhizal fungi (65%). In summary, the effects of this strain on the accumulation of different osmolytes were detected in mycorrhizal plants (Table 2), and this strain offered cellular protection against stress.

It is worth noting that despite the lower increases in proline and total soluble carbohydrate contents in the roots of mycorrhized plants under salt stress, a positive effect of mycorrhization on the concentration of these osmolytes was observed, which may have a direct impact on the osmotic adjustment of the plants. The response of these variables in stressed and inoculated plants indicates a beneficial role of *R. irregularis* in enhancing salt stress tolerance by contributing to cell turgor maintenance. Increased accumulation of these compatible organic solutes is one of the most important responses in crops for salt stress tolerance.

A combined analysis of the presented variables revealed that plants inoculated with the AMF strain INCAM 11, which was effective under the study conditions, underwent osmotic adjustment. This could be due, in part, to the accumulation of osmotically active organic solutes (Table 2; Fig. 2), which are manifested under these conditions as defense mechanisms to better tolerate NaCl salt stress.

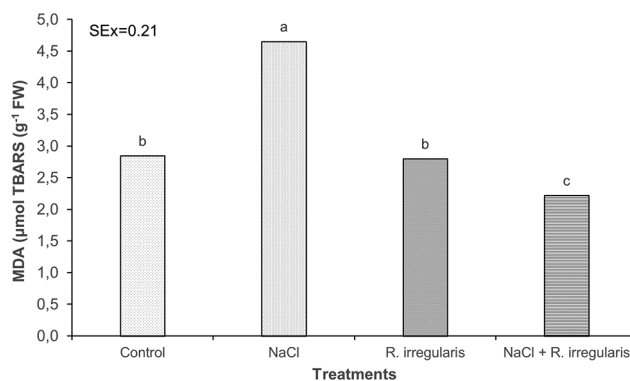


Fig. 3 Effects of mycorrhizal inoculation on lipid peroxidation (malondialdehyde) in the leaves of chickpea cv. 'N-29' plants exposed to 50 mM NaCl 35 days after sowing. Means with the same letters do not differ significantly from each other according to Duncan's multiple range test ($p \leq 0.05$) ($n=9$)

Oxidative metabolism

Lipid peroxidation

Figure 3 shows the malondialdehyde (MDA) concentrations in chickpea leaves exposed to NaCl and inoculated with AMF. Plants subjected to salinity without inoculation exhibited the highest MDA concentrations, while those with mycorrhization under saline conditions showed lower MDA levels, even compared to the control treatments.

The reduced MDA concentration in stressed, inoculated plants may be attributed to enhanced antioxidant activity, which helps mitigate oxidative damage. These results confirm those found in chickpea (*C. arietinum*) (Garg and Bhandari 2016b), pigeon pea (*Cajanus cajan*) (Pandey and Garg 2017) and cucumber (*C. sativus*) (Hashem et al. 2018). Similarly, Qin et al. (2021) reported that the MDA concentration decreased in mycorrhizal peanut plants relative to that in nonmycorrhizal plants.

Antioxidant enzyme activity

Table 3 shows the responses of several enzymes of the antioxidant system of chickpea plants (cv. 'N-29') subjected to salt stress. The activity of peroxidases was not affected by salt stress; only after inoculation with the AMF strain was a decrease observed. Catalase activity increased significantly in the salt-stressed and salt-inoculated treatments, while activity decreased significantly in the no-inoculated salt treatment. SOD activity increased in the saline treatments, and this response was greater in the uninoculated saline treatment. No significant differences were found between the control and no-inoculated treatments for CAT and SOD enzymes.

Table 3 Effects of mycorrhizal inoculation with *R. irregularis* on the specific activity of the antioxidant enzymes peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) in the leaves of Chickpea Cv. 'N-29' plants exposed to 50 mM NaCl 35 days after sowing

Treatments	POX ($\mu\text{mol H}_2\text{O}_2$ $\text{min}^{-1} \text{mg}^{-1}$ prot)	CAT ($\mu\text{mol H}_2\text{O}_2$ $\text{min}^{-1} \text{mg}^{-1}$ prot)	SOD UAE $\text{mg}^{-1} \text{prot}$
Control	0,06 a	0,457 b	92,27 c
50 mM NaCl	0,05 a	0,173 c	186,60 a
<i>R. irregularis</i>	0,04 b	0,363 b	92,60 c
<i>R. irregularis</i> + 50 mM NaCl	0,04 b	0,782 a	127,70 b
SE _x	$\pm 0,002$ ***	$\pm 0,07$ ***	$\pm 11,60$ ***

Means with equal letters in each column do not differ significantly from each other according to Duncan's multiple range test ($p \leq 0.05$) ($n=9$). SE_x: Standard error of the mean

Another antioxidant system in plants is the ascorbate-glutathione cycle (Skwarek-Fadecka et al. 2024). This is the main system for the detoxification of H_2O_2 in chloroplasts, mitochondria and the cytosol of plant cells. Four enzymes and two antioxidant compounds, ascorbate and glutathione participate in this cycle. Figure 4 shows the specific activities of ascorbate peroxidase and glutathione reductase enzymes in the leaves of chickpea plants inoculated with and exposed to NaCl. APX enzyme activity decreased with stress and with inoculation of the AMF strain and was significantly lower in the salinity and inoculated treatments.

Glutathione reductase activity decreased with mycorrhization, but increased with applied salt stress, being greater in stressed (50 mM NaCl) plants, followed by inoculated stressed (50 mM NaCl + *R. irregularis*) plants. Other authors reported increased SOD, CAT and GR activity in mycorrhizal stressed chickpea plants (Garg and Bhandari 2016a; Bharti and Garg et al. 2019).

Cheema and Garg (2022) reported that mycorrhizal inoculation helps chickpea plants survive under stress conditions by inducing an antioxidant system as a defense

mechanism. They also reported that mycorrhizal chickpea plants decreased O_2 and H_2O_2 due to the enhanced activity of SOD, CAT and POX, indicating the potent role of AMF in reducing oxidative damage in chickpea in a specific manner. In this work, compared with nonmycorrhizal plants, mycorrhizal plants only showed a significant increase in the specific activity of the CAT enzyme under saline conditions. In the cases of SOD and GR, increases were observed, but with lower values than those exhibited by nonmycorrhizal plants. POX activity did not change under stress.

Abscisic acid (ABA) and gibberellic acid (GA) content

Phytohormones regulate plant growth and development under both normal and stress conditions. Phytohormones such as abscisic acid (ABA) and gibberellic acid (GA_3) play a role in salt stress tolerance in plants (Fahad et al. 2015).

Figure 5 shows the concentrations of ABA and GA_3 in inoculated and NaCl-stressed chickpea plants, which behaved similarly. The ABA and GA_3 contents increased with salinity, with the highest values found in the plants in the uninoculated saline treatment (50 mM NaCl), followed by those in the inoculated stressed plants (50 mM NaCl + *R. irregularis*). During osmotic stress, cellular dehydration occurs, and plants respond by exhibiting a wide variety of responses involving ABA, ranging from rapid physiological changes such as stomatal closure to the prevention of water loss in plants or changes at the cellular level where ABA can regulate a broad set of genes to promote tolerance (Ren et al. 2018).

The higher concentration of ABA in the non-stressed inoculated treatment (*R. irregularis*) than in the control (Fig. 5) could be explained by the fact that *R. irregularis* may synthesize or excrete ABA located in the spores and in the extra and intraradical hyphae (Esch et al. 1994). Moreover, an increase in its concentration has been detected during mycorrhization (Ludwig 2010), and it is thought

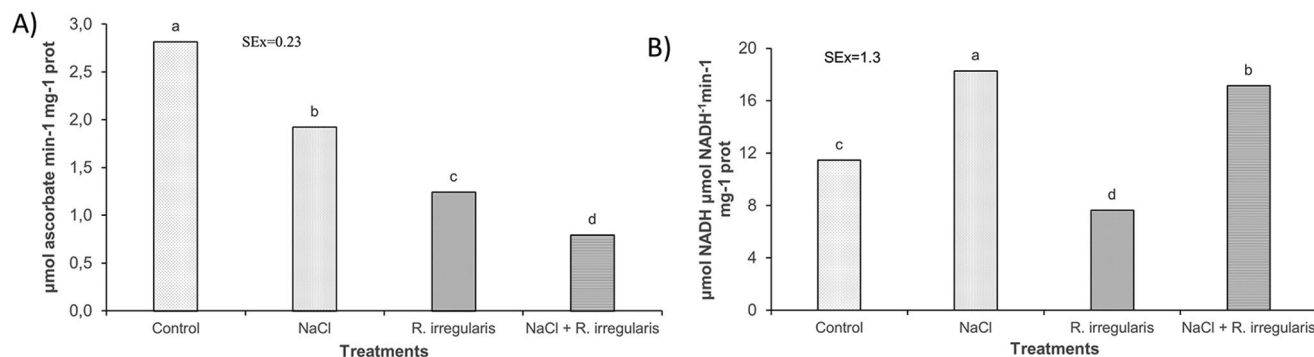


Fig. 4 Effects of mycorrhizal inoculation on the specific activity of several enzymes of the Ascorbate-Glutathione Cycle of chickpea cv. 'N-29' plants stressed by NaCl 35 days after sowing. **A)** APX: Ascorbate peroxidase. **B)** GR: Glutathione reductase. Means with the same

letters do not differ significantly from each other according to Duncan's multiple range test ($p \leq 0.05$) ($n=9$). SE_x: standard error of the mean

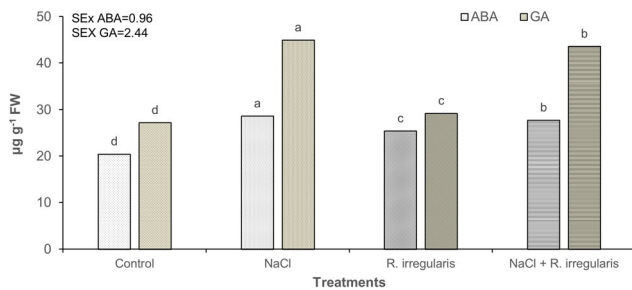


Fig. 5 Effects of mycorrhizal inoculation on abscisic acid (ABA) and gibberellic acid (GA3) concentrations in the leaves of chickpea cv. 'N-29' plants exposed to 50 mM NaCl 35 days after sowing. Means with the same letters do not differ significantly from each other according to Duncan's multiple range test ($p \leq 0.05$) ($n=9$). *SE*x: Standard error of the mean

to be involved in susceptibility to colonization and in the development and functionality of arbuscules (Herrera et al. 2007). Although mycorrhization favors ABA concentration, in this study, it was observed that under saline conditions, the increase in ABA concentration was lower than that in stressed plants without inoculation. Similarly, Hashem et al. (2018) reported a decrease in ABA concentrations in stressed and inoculated cucumber (*C. sativus*) plants compared with those in stressed non-inoculated plants.

Mycorrhizal colonization has a positive influence on GA concentrations₃ in plants (Shaul et al. 2002). In crops such as tomato and chickpea, gibberellins have been shown to induce salinity tolerance (Khalloufi et al. 2017; Garg and Bharti 2018). With exogenous applications of GA₃ in tomato (*S. lycopersicum*), relatively high nutrient concentrations were detected, indicating that GA₃ promotes salinity tolerance in crops (Khalloufi et al. 2017). The levels of plant hormones such as ABA, auxins, and GA₃, among others, change after the establishment of mycorrhization (Hause et al. 2007), suggesting an alteration in the hormonal balance in mycorrhized plants. Mycorrhizal plants increase water levels when exposed to salt stress and maintain cell turgor, photosynthesis and enzyme activity.

Osmolyte production is a plant protection mechanism under stress conditions. All of these genes play important roles in salinity conditions, controlling osmotic adjustment and acting as stress signals in plants grown under saline conditions (Hashem et al. 2018). Proline is an osmoprotectant that prevents oxidative damage to cells, maintaining cellular water potential and protein integrity and function (Mansour and Ali 2017). In this study, mycorrhizal- and nonmycorrhizal-stressed plants had higher concentrations of proline, glycine betaine and glutamate in their leaves.

When plants are exposed to salt stress, reactive oxygen species such as H₂O₂ and O₂⁻ are accumulated. These reactive oxygen species are often associated with antioxidant enzymes of the defense system for avoid the oxidative

damage, (Nisar et al. 2019). To maintain membrane stability and permeability, lipid peroxidation decreased in stressed and inoculated chickpea plants, and it was inferred that these plants decreased the accumulation of ROS under stress conditions, thus improving their antioxidant defense mechanisms via CAT activity.

Despite the reductions in the concentrations of POX, SOD (Table 3) and GR (Fig. 4) in the inoculated stressed plants compared with those in the non-inoculated stressed plants, these changes helped to reduce cellular oxidative damage. Notably, mycorrhizal inoculation decreases ROS accumulation and prevents oxidative cell damage (Huang et al. 2017).

Mycorrhizal fungi associated with plants regulate stress hormone signaling under unfavorable environmental conditions. In this study, the ABA and GA concentrations₃ were greater in the stressed plants than in the inoculated stressed plants. Moreover, ABA acts as an antitranspirant, leading to a reduction in water loss through the modification of stomatal function. In the present study, the plants inoculated with AMF also exhibited increased concentrations of these phytohormones under saline conditions; however, this increase was lower than that observed in non-inoculated stressed plants.

Conclusions

Plants inoculated with *R. irregularis* exhibited improved salinity tolerance, primarily through enhanced antioxidant activity and osmotic regulation. Salt stress induced a redox imbalance, resulting in reductions of 35%, 20%, and 25% in POX, SOD, and APX activity, respectively, while increasing free radical levels. However, mycorrhizal inoculation under 50 mM NaCl conditions led to increased total soluble carbohydrate accumulation and enhanced catalase and glutathione reductase activity, contributing to redox homeostasis. These findings provide valuable insights into the role of *R. irregularis* in mitigating salinity stress in chickpea. Future research should investigate molecular mechanisms, particularly gene expression related to salinity tolerance, to enhance our understanding of AMF-mediated stress responses.

Further studies should also assess the performance of multiple AMF strains under field conditions over an extended growth period to validate these findings in practical agricultural settings. Integrating molecular analyses, such as transcriptomic and proteomic studies, could further clarify the pathways involved in AMF-induced salinity tolerance. Addressing these aspects will strengthen the application of AMF in sustainable agriculture, potentially

leading to improved crop resilience and productivity under salinity stress.

Author contributions L.A.M, Y.M.H., and O.P.R., conceptualized the study, conducted the experiments and prepared the manuscript and performed biochemical analyses. Y.R.Y. and J.M.D.A.M. contributed to formatting. U.A. and J.H.S. coordinated the research and reviewed the manuscript. All authors read and approved the final manuscript.

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Code availability Not applicable.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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